



KONGERIKET NORGE  
The Kingdom of Norway

Rec'd PCT/PTO

09 MAY 2005

10/511960

Bekreftelse på patentsøknad nr  
*Certification of patent application no*



20023357

▷ Det bekreftes herved at vedheftede dokument er nøyaktig utskrift/kopi av ovennevnte søknad, som opprinnelig inngitt 2002.07.11

▷ It is hereby certified that the annexed document is a true copy of the above-mentioned application, as originally filed on 2002.07.11

Priority is claimed from patent application no 20021886 filed on 2002.04.19

2004.10.20

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## Søknad om patent

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02-07-11\*20023357

Skal utfylles av Patentstyret

Behandlende medlem EN

Int. Cl. G 01 N

Alm. tilgj. 20 OKT 2003

Søkers/fullmektigens referanse  
(angis hvis ønsket):

PN0222-NO1

Oppfinnelsens  
benevnelse:

Blanding

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Prioritet kreves fra dato 19.04.2002 sted Norge nr. 20021886

Prioritet kreves fra dato ..... sted ..... nr. ....

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Mixture

The invention relates to methods for determining protein activity using NMR spectroscopy. The present invention provides a method for determining protein activity *in vivo* using probe compounds and enhancing the nuclear polarisation of NMR active nuclei present in the probe compounds (hereinafter termed "hyperpolarisation") prior to NMR analysis. The invention also provides mixtures of probe compounds for the above-mentioned method.

- 10 The ability of an organism to absorb a drug, translocate it, break it down (metabolise it) and finally remove it from the organism itself is crucial to how well a drug will operate in a particular organism or individual. For clinical trialing of a new drug as well as for the therapeutic efficacy of a drug it is important to gain better understanding of the performance of a drug in a human individual or human population. Thus, an attempt was made to classify populations into groups of individuals with similar biological characteristics and behaviours. This attempt has become known as phenotyping. In the context of the present invention, a phenotype is defined in one of the three distinct ways: i) the totality of the observable functional and structural characteristics of an organism as determined by interaction of the genotype of the organism with the environment in which it exists, ii) any particular characteristic or set of characteristics of a organism so determined and iii) a group of organisms exhibiting the same set of such characteristics.

- 25 Clinical trialing of a new drug in the human population is an expensive and protracted process. Late failure of a putative drug has a significant impact on the profitability of the developer, while withdrawal of a drug after its launch on the open market has an even greater impact on the valuation and reputation of a pharmaceutical company. Phenotyping of a clinical trial group is therefore potentially very valuable in understanding how individuals respond beneficially or adversely to a new drug. Using volunteer patients for clinical trials of defined phenotypes facilitates the design of clinical phase I and II protocols and the interpretation of clinical data and potential adverse drug reactions during the trial can be reduced.

Therapeutic efficacy of a drug is dependent on if and how individuals respond to the administered drug. On the basis of the extent to which a therapeutic drug is metabolised, individuals might be characterised as being extensive, normal or poor metabolisers of a therapeutic drug.

5

In normal metabolisers, steady-state drug levels are within the expected therapeutic range and toxic effects are absent whilst in extensive metabolisers, steady-state drug levels are sub-therapeutic which can lead to no drug effect at all. In poor metabolisers, steady-state drug levels are larger than expected and these individuals are thus susceptible to undesired toxicity or other adverse effects of the drug. Thus, phenotyping of an individual receiving therapeutic drug treatment is valuable in understanding how individuals respond to certain drugs and drug doses and it is potentially helpful in determining adequate drugs and drug doses in order to achieve optimal therapeutic results.

15

Metabolism and transport of drug molecules in the human or non-human animate body are governed by certain proteins, e.g. enzymes or transporter proteins. The determination of the activity of said proteins can be used to phenotype individuals. Cytochrome P 450 (CYP450) plays a key role in the metabolism of drugs. The members of the CYP450 superfamily of oxidases show a common catalytic mechanism but individual isoenzymes have divergent substrate specificity. In order to assess the multiplicity of the CYP450 isoenzymes it is not possible to study metabolism with a single probe compound but several probe compounds which act as substrates for different CYP450 isoenzymes have to be used.

25

R. J. Scott et al., Rapid Commun. Mass Spectrom. 13, 1999, 2305-2319 describe the use of a "cocktail" of multiple probe drugs for studying the *in vitro* metabolism of said cocktail in human urine or plasma samples upon addition of the enzyme  $\beta$ -glucuronidase. After reaction, the samples were worked up by solid phase extraction and analysed by liquid chromatography and mass spectrometry (LC/MS/MS). The disadvantage of this method is that work-up of the samples is time consuming. Moreover, due to reduced recovery of the probe drugs and their metabolites after solid phase extraction it might be difficult to detect small amounts of metabolites.

30

Another disadvantage is that any small change in the method itself requires a careful validation.

In WO-A-00/35900 several probe drugs comprising phenolic dyes are used for *in vitro* screening assays as optical probes or sensors of the activity of CYP450 isoenzymes. The disadvantage with this method is that the addition of dye may influence the metabolic breakdown. Moreover, optical measurements may not be sufficiently specific due to, e.g. dye leakage, dye compartmentalisation or quenching of signals. Due to the lack of specificity of current dyes, the method requires the use of single expressed isoenzymes. Although the method provides an indication of potential drug-drug interactions, it is far removed from the real *in vivo* situation. Thus, the method can only be employed as an initial screening method.

K. Akira et al., Drug Metab. Dispos 29, 2001, 903-907 describe the use of  $^{13}\text{C}$ -labelled antipyrine as an *in vivo* probe to evaluate some CYP450 isoenzymes using  $^{13}\text{C}$ -NMR spectroscopy. The disadvantage of this method is that only a small number of CYP450 isoenzymes are addressed by using a single probe. Due to the reduced sensitivity of conventional  $^{13}\text{C}$ -NMR spectroscopy, the probe drug has to be administered in large amounts leading to potential risk of adverse drug effects in the patients.

WO-A-01/96895 describes a method for obtaining information regarding the fate of a test compound in a biological system by enhancing the nuclear polarisation of an NMR active nuclei present in the test compound (hyperpolarisation) prior to NMR analysis. The disadvantage of this method is that only one test compound is used. Thus, metabolic key pathways like the CYP450 metabolism of drug compounds which are especially useful for phenotyping of individuals can not be addressed in one experiment.

Thus, there was a need for a fast and simple method for determining the activity of proteins responsible for the transport and metabolism *in vivo* which allows phenotyping of individuals.

The present invention provides a method for determining *in vivo* protein activity comprising

- a) selecting at least two probe compounds each containing at least one NMR active nuclei
- 5 b) administering said probe compounds to a human or non human animate body
- c) collecting samples from said human or non human animate body
- d) hyperpolarising the NMR active nuclei of said samples, and
- e) analysing said samples by NMR spectroscopy.

10 In the context of the present invention, "proteins" means all proteins whose activity can be influenced by a probe compound acting e.g. as a substrate, inducer or inhibitor of said protein. Preferred proteins are enzymes and transporter proteins, e.g. NADPH quinone oxireductases, preferably CYP450, N-acetyltransferase, glutathione transferase, serotonin transport protein, and p-glycoprotein.

15 The data acquired in step e) can be used to determine protein activity in a number of ways. e.g. by determining the rate of disappearance of the probe compound from biofluid samples like urine or plasma with time. As this has been a difficult and time consuming task, calculating the ratio of the probe compound to their metabolites at  
20 one or more selected time points is preferred (determination of metabolic ratio).

In step a) of the method according to the invention, at least two probe compounds are selected, each of the probe compounds contain at least one NMR active nuclei.

25 Suitably, at least 3 probe compounds are used in the method of the invention, most suitably at least 4 and preferably at least 7 probe compounds.

The selection according to step a) is dependent on which protein activity is going to be determined, e.g. which enzyme / enzyme family and which isoenzymes of said  
30 enzyme family will be addressed. The selection should be done in such a way that preferably all or at least most of the isoenzymes of a certain enzyme family should be addressed with specific probe compounds. Preferably, probe compounds and their metabolites show a well-dispersed NMR spectrum in order to clearly distinguish between the single probe compounds and their metabolites.

Preferably, safety and availability of the probe compounds are high. It is further preferred that the probe compounds are selected so that it is possible to analyse the probe compounds and their metabolites in different samples collected from a human or non human animate body, particularly in different biofluids.

If the enzyme family to be addressed in the method according to the invention is CYP450, a number of possible probe compounds for different isoenzymes are known (see for example R. J. Scott et al., Rapid Commun. Mass Spectrom. 13, 1999, 2305-2319 or R.F. Frye et al., Clin. Pharmacol. Ther. 62, 1997, 365). Said probe compounds are preferably selected according to the above-mentioned aspects.

Suitably, the probe compounds are substrates for CYP450, preferably substrates for CYP450 isoenzymes selected from the group consisting of CYP1A2, CYP2A6, CYP2B6, CYP2C8/9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4.

Preferably, the probe compounds are substrates for CYP450 selected from the group consisting of Phenacetin, Coumarin, Tolbutamide, Mephénytoin, S-Mephénytoin, Bufuralol, Chlorzoxazone, Midazolam, Caffeine, Dapsone, Diclofenac, Debrisoquine, Bupropion, Antipyrine and Dexomethorphan.

The probe compounds according to the invention contain at least one NMR active nuclei, i.e. a nuclei with non-zero nuclear spin. Preferred nuclei are  $^{13}\text{C}$ ,  $^{15}\text{N}$ ,  $^{31}\text{P}$ ,  $^{19}\text{F}$ , and/or  $^1\text{H}$ . Isotopically enriched probe compounds can be employed. If non-enriched probe compounds are employed, probe compounds containing nuclear species occurring at high natural abundance such as  $^{31}\text{P}$ ,  $^{19}\text{F}$ , and/or  $^1\text{H}$  are employed. However, isotopically enriched probe compounds, preferably enriched with non-radioactive isotopes, are preferably used in the method according to the invention as the isotopic enrichment has substantially no effect on the therapeutic efficacy of the probe compound and the NMR detection is strongly facilitated.

The enrichment may include either selective enrichments of one or more sites within the probe compound molecule or uniform enrichment of all sites. Preferably, the probe compounds are isotopically enriched in only one position of the molecule.

Enrichment can be achieved by chemical synthesis or biological labelling. Suitably, a probe compound for use in the method according to the invention is an organic compound isotopically enriched in only one position of the molecule with an enrichment of at least 10%, most suitably at least 25%, preferably at least 75%, most  
5 preferably at least 90%, ideally approaching 100%.

In a preferred embodiment of the present invention, the probe compounds are enriched with  $^{13}\text{C}$  and/or  $^{15}\text{N}$ , preferably with  $^{13}\text{C}$  or  $^{15}\text{N}$ , particularly preferred with  $^{13}\text{C}$  as for higher sensitivity and a broader choice of labelling. In a further preferred  
10 embodiment, all probe compounds are enriched with the same NMR active nuclei. Thus, it is possible to collect information in a single NMR analysis.

The optimal position for isotopic enrichment in the probe compound is dependent on the relaxation time of the NMR active nuclei. Preferably, probe compounds are  
15 isotopically enriched in positions with long T1 relaxation time. Further, the probe compounds are preferably isotopically enriched at positions in the molecule where upon metabolism structural changes take place. This leads to greater chemical shift differences between the probe compound and its metabolites which lead to better dispersed NMR spectra. Labelling in two or more positions may facilitate the  
20 interpretation of complex NMR spectra.

In step b) of the method of the invention, the probe compounds are administered to a human or non human animate body.

25 The probe compounds can either be administered sequentially or as a mixture of probe compounds.

If the probe compounds are administered in a mixture, probe compounds can be mixed and subsequently dissolved or dispersed in a solvent or a solvent mixture  
30 which can then be directly used for administration or which can be further treated before the administration. Alternatively, each probe compound or some of the probe compounds are dissolved or dispersed in a solvent or a solvent mixture first and then a mixture of the dissolved / dispersed probe compounds is prepared. In order to achieve proper mixing the usual mixing techniques such as stirring, bubbling,  
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agitation, vortexing or sonification. In another embodiment, mixtures of solid probe compounds are provided.

5 The solvents or solvent mixtures used for dissolving or dispersing the probe compounds are preferably solvents which can be used in connection with administration to a human or non-human animate body.

10 For administration, the probe compounds are preferably formulated in conventional pharmaceutical or veterinary administration forms. If the probe compounds are administered in solution than they may be in the form of a suspension, dispersion, slurry etc., for example in an aqueous vehicle such as water. If the probe compounds are administered in solid form, than they may be in the form of tablets or powder.

15 For administration, the probe compounds may further contain pharmaceutically acceptable diluents and excipients and formulation aids e.g. stabilisers, antioxidants, osmolality adjusting agents, buffers or pH-adjusting agents. For injection, a sterile solution or suspension of the probe compounds is most preferred. For parental administration, a carrier medium which is preferably isotonic or somewhat hypertonic, is preferred.

20 The probe compounds are preferably administered into the vasculature or directly into an organ or muscle tissue as well as subdermally or subcutaneously. In another preferred embodiment, the probe compounds are administered via non-parental route such as transdermal, nasal, sub-lingual or into an external body cavity e.g. orally in  
25 the gastro-intestinal tract.

The dosage for administration is preferably therapeutic or sub-therapeutic, particularly preferably sub-therapeutic. Due to the sensitivity of the method according to the invention, sub-therapeutic administration is possible which strongly  
30 minimises the risk of adverse effects of the probe compounds.

In step c) of the method of the invention, samples from said human or non-human animate body are collected. Samples may be taken once, at time intervals or continuously (dynamic studies)

Samples that may be collected include tissue or cell samples, faeces, biofluids including but not limited to blood, blood plasma, lymph, urine, semen, breast milk, cerebro-spinal fluid, sweat, lachrymal or parotid secretions or lavage. Preferably,  
5 samples collected are biofluids, particularly preferably blood, blood plasma or urine.

If the method according to the invention is used for determining the *in vivo* activity of CYP450 isoenzymes, collected samples from human or non-human animate bodies are preferably blood, blood plasma and urine.

10

The collected samples may be further processed, e.g. in order to separate cells from liquids. Thus, blood may be treated in order to obtain blood plasma. The samples may be purified prior to hyperpolarisation and/or analysis but this is not always necessary. An important advantage of the method according to the invention is that  
15 analysis can be carried out directly on the crude sample without the need for fractionation, purification or concentration steps.

20

If the protein activity is determined by calculating the rate of disappearance of the probe compounds, a reference standard may conveniently be included in the sample before hyperpolarisation. Inclusion of said standard allows the determination of the concentration of the probe compounds and their metabolites. Preferably, one standard is added. Suitable standards are simple molecules comprising signals which do not interfere with the signals from the probe compounds and their metabolites. Preferred standards do comprise only one signal. Conveniently, a chemical shift  
25 reference is added to the sample before hyperpolarisation.

30

In step d) of the method of the invention, the NMR active nuclei of said samples are hyperpolarised.

There are several ways for hyperpolarising NMR active nuclei, preferred ways are polarisation transfer from a noble gas, "brute force", DNP and spin refrigeration, all explained below.

A preferred way for hyperpolarising the NMR active nuclei containing probe compounds according to the invention is the polarisation transfer from a hyperpolarised noble gas. Noble gases having non-zero nuclear spin can be hyperpolarised, i.e. have their polarisation enhanced over the equilibrium polarisation, e.g. by the use of circularly polarised light. A hyperpolarised noble gas, preferably  $^3\text{He}$  or  $^{129}\text{Xe}$ , or a mixture of such gases, may be used according to the present invention to effect hyperpolarisation of the NMR active nuclei present in the probe and/or test compounds. The hyperpolarisation may also be achieved by using an artificially enriched hyperpolarised noble gas, preferably  $^3\text{He}$  or  $^{129}\text{Xe}$ . The hyperpolarised gas may be in the gas phase, it may be dissolved in a liquid, or the hyperpolarised gas itself may serve as a solvent. Alternatively, the gas may be condensed onto a cooled solid surface and used in this form, or allowed to sublime. Either of these methods may allow the necessary intimate mixing of the hyperpolarised gas with the target to occur. In some cases, liposomes or microbubbles may encapsulate the hyperpolarised noble gas.

Another preferred way for hyperpolarising the NMR active nuclei containing probe compounds according to the invention is that polarisation is imparted to said NMR active nuclei by thermodynamic equilibration at a very low temperature and high field. Hyperpolarisation compared to the operating field and temperature of the NMR spectrometer is effected by use of a very high field and very low temperature (brute force). The magnetic field strength used should be as high as possible, suitably higher than 1 T, preferably higher than 5 T, more preferably 15 T or more and especially preferably 20 T or more. The temperature should be very low, e.g. 4.2 K or less, preferably 1.5 K or less, more preferably 1.0 K or less, especially preferably 100 mK or less.

Another preferred way for hyperpolarising the NMR active nuclei containing probe compounds according to the invention is the DNP (dynamic nuclear polarisation) method effected by a DNP agent. DNP mechanisms include the Overhauser effect, the so-called solid effect and the thermal mixing effect. Most known paramagnetic compounds may be used as DNP agents, e.g. transition metals such as chromium (V) ions, magnesium (II) ions, organic free radicals such as nitroxide radicals and trityl radicals (WO-A-98/58272) or other particles having associated free electrons.

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Preferably, radicals with low relaxivity are used as DNP agents. Where the DNP agent is a paramagnetic free radical, the radical may be conveniently prepared *in situ* from a stable radical precursor by a radical-generating step shortly before the polarisation, or alternatively by the use of ionising radiation. During the DNP process, energy, normally in the form of microwave radiation, is provided, which will initially excite the paramagnetic species. Upon decay to the ground state, there is a transfer of polarisation to the NMR active nuclei of the target material. The method may utilise a moderate or high magnetic field at a very low temperature, e.g. by carrying out the DNP process in liquid helium and a magnetic field of about 1 T or above. Alternatively, a moderate magnetic field and any temperature at which sufficient NMR enhancement is achieved in order to enable the desired studies to be carried out may be employed. The method may be carried out by using a first magnet for providing the polarising magnetic field and a second magnet for providing the primary field for MR spectroscopy.

Another preferred way for hyperpolarising the NMR active nuclei containing probe and/or test compounds according to the invention is the spin refrigeration method. This method covers spin polarisation of a solid compound or system by spin refrigeration polarisation. The system is doped with or intimately mixed with suitable paramagnetic materials such as  $\text{Ni}^{2+}$ , lanthanide or actinide ions in crystal form with a symmetry axis of order three or more. The instrumentation is simpler than required for DNP with no need for a uniform magnetic field since no resonance excitation field is applied. The process is carried out by physically rotating the sample around an axis perpendicular to the direction of the magnetic field. The prerequisite for this method is that the paramagnetic species has a highly anisotropic g-factor. As a result of the sample rotation, the electron paramagnetic resonance will be brought in contact with the nuclear spins, leading to a decrease in the nuclear spin temperature. Sample rotation is carried out until the nuclear spin polarisation has reached a new equilibrium.

Some of the hyperpolarisation techniques described above, e.g. DNP, brute force or spin refrigeration transfer, are only effective when transferring polarisation to the solid state. If the sample is not solid, it may conventionally be frozen in an appropriate solvent or solvent mixture prior to hyperpolarisation by one of the

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methods that needs to be carried out in the solid state. Solvent mixtures have been found to be particularly suitable, especially if the mixture forms an amorphous glass, preferably by use of glycerol. Such a matrix of amorphous glass is preferably employed in DNP hyperpolarisation to ensure homogenous intimate mixing of radical and target in the solid.

The degree of hyperpolarisation of the NMR active nuclei according to the invention can be measured by its enhancement factor compared to thermal equilibrium at spectrometer field and temperature. Suitably the enhancement factor is at least 10, preferably at least 50 and more preferably at least 100. However, methods according to the invention where even smaller enhancements are achieved may still be performed usefully due to the shorter time needed for the total measurement compared with methods described in the prior art. If the enhancement is reproducible and the hyperpolarisation/NMR analysis can be repeated, the signal to noise ratio of a NMR signal can be improved. In such a case, the minimum NMR enhancement factor required depends on the hyperpolarisation technique and the concentration of the probe/test compound and their metabolites. The enhancement has to be large enough so that the NMR signal from the probe/test compound and their metabolites can be detected. In this context, it is clear that an enhancement of 10 or less than 10 that is achievable in a multi-shot experiment may be very useful due to the time saved in data acquisition compared with conventional NMR.

In step e) of the method of the invention the samples from step d) are analysed by NMR spectroscopy. The analysis may be carried out by continuous monitoring or as a single discrete measurement or as a series of discrete measurements that may be carried out at suitable intervals over time. Thus, it is possible to identify many and preferably all changes in metabolism and appearance of individual metabolites of the probe compounds.

The hyperpolarised sample may as well be further diluted or mixed with suitable solvents or solvent mixtures, for NMR spectroscopy, depending on which kind of NMR analysis, e.g. liquid or solid phase NMR spectroscopy has to be applied.

After hyperpolarisation, it is desirable to preserve as much as possible of the polarisation prior to NMR analysis. Some of the hyperpolarisation techniques described above, e.g. by DNP, brute force, spin refrigeration transfer, are only effective when transferring polarisation to the solid state. However, it is often desired to investigate the NMR spectrum of a sample in the liquid state, in order to improve spectral resolution and sensitivity. Alternatively, line-narrowing techniques like Magic Angle Spinning (MAS) can be employed to increase spectral resolution of NMR in the solid state and enable low temperature NMR analysis.

If a liquid state NMR technique is to be employed, once the sample has been hyperpolarised, it can be rapidly removed from the polarisation chamber and then dissolved in a suitable solvent. It is advantageous to use solvents which do not interfere with the spectra produced in the analysis step or solvents which keep a stable chemical environment and prolong the T1 relaxation time. Deuterated solvents such as D<sub>2</sub>O or mixtures of methanol and acid, preferably with an excess of methanol, are particularly suitable. Stirring, bubbling, sonification or other known techniques can be used to improve the speed of dissolution. Suitably, the temperature and the pH of the solution are maintained to allow optimal dissolution and a long nuclear relaxation time.

Preferably, the sample or a solution thereof is kept in a holding field throughout the period between polarisation and analysis in order to prevent relaxation. A holding field provides a higher field than the Earth's magnetic field and suitably higher than 10 mT. It is suitably uniform in the region of the sample and the optimal conditions will depend on the nature of the sample.

The sample or a solution thereof is subsequently transferred for examination by standard solution phase NMR analysis. The transfer process is manually or automated, preferably automated. Alternatively, the hyperpolarisation step and optional subsequent dissolution steps are suitably integrated into a single automated unit. In an additional suitable embodiment, hyperpolarisation and optional dissolution steps are automated and NMR detection hardware is also housed within the same single fully integrated unit.

Alternatively, where a solid state NMR technique is to be used, the solid state sample may be hyperpolarised, e.g. by DNP, brute force, spin refrigeration transfer or any other method that will work in the solid state at low temperature. Subsequently, the hyperpolarised sample will be moved into a solid state MAS NMR probe. The movement is suitably rapid and is preferably carried out via lifting or ejection. The sample in the NMR probe will then be spun so that high resolution solid state NMR spectroscopy can be carried out. The entire process can be automated and will preferably be carried out in an integrated unit.

10 In a preferred method of the invention, the *in vivo* activity of CYP450 is determined by:

- a) selecting at least 3  $^{13}\text{C}$ -isotopically enriched probe compounds from the group consisting of Phenacetin, Coumarin, Tolbutamide, Mephénytoin, S-Mephénytoin, Bufuralol, Chlorzoxazone, Midazolam, Caffeine, Dapsone,  
15 Diclofenac, Debrisoquine, Bupropion, Antipyrine and Dexomethorphan, the probe compounds being isotopically enriched in positions with long T1 relaxation time
- b) administering said probe compounds to a human or non human animate body
- c) collecting urine samples and/or blood samples of said human or non human  
20 animate body
- d) hyperpolarising the NMR active nuclei of said samples using the DNP method and
- e) analysing the samples by  $^{13}\text{C}$ -NMR spectroscopy.

25 The method of the invention can be used for phenotyping of individuals, e.g. for phenotyping of a clinical trial group or for phenotyping of individuals who will receive therapeutic drug treatment. If the method according to the invention is used for phenotyping of a clinical trial group, protein activity according to the invention is determined in the volunteer patients. According to the data and information gained  
30 by the method of the invention, volunteer patients are classified into groups of individuals showing similar biological behaviour towards the probe compounds. If the method according to the invention is used for phenotyping of an individual who will receive therapeutic drug treatment, the data and information gained by the

method of the invention may be compared to data and information - gained by the method of the invention - obtained from other individuals.

If the method according to the invention is used for phenotyping, protein activity, e.g. enzyme activity may be determined by calculating the metabolic ratio between the probe compounds and their metabolites. In order to evaluate a metabolic ratio from a particular individual, it may be compared to a statistical material. The statistical material may be obtained by calculating the metabolic ratio between probe compounds and their metabolites in a large number of individuals. A frequency distribution histogram may be established (number of individuals vs. metabolic ratio). If e.g. a polymorphism is present in the enzymatic pathway under evaluation, then the distribution will be bimodal. This bimodal distribution reflects that a subset of the population is unable to or suffers from some deficiency in metabolising the probe compounds through the particular enzymatic pathway. An antimode (a definitive separating value) will separate the individual modes of the distribution. Based on this bimodal population distribution it is possible to define a phenotype by being able to distinguish between two populations e.g. the poor metabolisers and the extensive metabolisers. The antimode serves as a threshold for distinguishing between the two phenotypes. Metabolic ratio values above the antimode will indicate a poor metaboliser whereas metabolic ratio values below the antimode will indicate an extensive metaboliser phenotype.

Another aspect of the invention is a method as described above wherein from said analysis of step e) an NMR pattern I is generated. Said NMR pattern is preferably stored electronically, e.g. in a database.

- Subsequently said method comprises the further steps of
- f) administering said probe compounds a) and at least one putative drug to a human or non human animate body
  - g) subsequently carry out steps c) and d)
  - h) analysing the samples collected in step d) by NMR spectroscopy and hereby generating an NMR pattern II
  - i) comparing the NMR patterns I and II thus identifying distinctions in the NMR pattern II which are due to the administration of the putative drug.



Steps f) to h) are carried out as described above and the NMR pattern II obtained in step h) is preferably stored electronically, e.g. in a database. The comparison of the NMR patterns I and II is preferably carried out using algorithmic analysis, typically  
5 by employing a computer with appropriate software.

The such acquired NMR patterns allow the determination of the change of *in vivo* protein activity upon administration of the probe compounds a) alone (NMR pattern I) and the administration of the probe compounds in combination with a putative  
10 drug (NMR pattern II). Thus, the possible influence of a putative drug can be determined.

This method may be used to study drug-drug interaction which is an important feature in the development of new drugs. The term "drug-drug interaction" refers to  
15 a new chemical entity being potentially interacting with already existing pharmacological drugs. As many diseases require treatment with multiple drugs, drug-drug interaction may result in adverse side effects which can lead to potential new drugs being withdrawn. Although various *in vitro* techniques have been introduced to evaluate the potential for such interactions, predicting the *in vivo*  
20 importance of such interactions proves to be difficult.

The above-mentioned method can be repeated for several single putative drugs or several putative drugs. Thus, a first NMR pattern related to a first set of probe compounds of a human or non human animate being can be stored and compared  
25 with subsequently obtained NMR patterns related to the first set of probe compounds in combination with several single putative drugs or several putative drugs.

Another aspect of the present invention is a method for determining *in vivo* protein activity, said method comprises  
30 a) collecting samples from a human or non human animate body preadministered with at least two probe compounds each containing at least one NMR active nuclei,  
b) hyperpolarising the NMR active nuclei of said samples and  
c) analysing said samples by NMR spectroscopy.

In a preferred embodiment, an NMR pattern I is generated in step c) which is preferably stored electronically, e.g. in a database. Subsequently said preferred embodiment comprises the further steps of

- 5 d) collecting samples from a human or non human animate body preadministered with at least two probe compounds of a) and at least one putative drug
- e) hyperpolarising the NMR active nuclei of said samples and
- f) analysing said samples by NMR spectroscopy and hereby generating an NMR pattern II
- 10 g) comparing the NMR patterns I and II thus identifying distinctions in the NMR pattern II which are due to the administration of the putative drug

The methods of the invention are preferably used for determining the *in vivo* activity of cytochrome P 450 (CYP450).

15

In a preferred embodiment, the probe compounds are substrates for CYP450, preferably substrates for CYP450 isoenzymes selected from the group consisting of CYP1A2, CYP2A6, CYP2B6, CYP2C8/9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4.

20

In a further preferred embodiment the probe compounds are substrates for CYP450 selected from the group consisting of Phenacetin, Coumarin, Tolbutamide, Mephentoin, S-Mephentoin, Bufuralol, Chlorzoxazone, Midazolam, Caffeine, Dapsone, Diclofenac, Debrisoquine, Bupropion, Antipyrine and Dexomethorphan.

25

Another aspect of the present invention is a mixture comprising at least two probe compounds, all probe compounds being enriched with  $^{13}\text{C}$  and/or  $^{15}\text{N}$  NMR active nuclei. Preferably, said mixture is used in the methods described above, particularly preferably for metabolic phenotyping.

30

Suitably, said mixture comprises at least 3 probe compounds, most suitably at least 4 and preferably at least 7 probe compounds.

In a preferred embodiment, the probe compounds in said mixture are enriched with  $^{13}\text{C}$  or  $^{15}\text{N}$ , particularly preferred with  $^{13}\text{C}$ . In a further preferred embodiment, the probe compounds in said mixture are enriched with the same NMR active nuclei.

5 In a preferred embodiment, said mixture comprises probe compounds which are substrates for CYP450, particularly preferably substrates for CYP450 isoenzymes selected from the group consisting of CYP1A2, CYP2A6, CYP2B6, CYP2C8/9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4.

10 In a further preferred embodiment, said mixture comprises probe compounds which are substrates for CYP450, particularly preferably substrates for CYP450 selected from the group consisting of Phenacetin, Coumarin, Tolbutamide, Mephenytoin, S-Mephenytoin, Bufuralol, Chlorzoxazone, Midazolam, Caffeine, Dapsone, Diclofenac, Debrisoquine, Bupropion, Antipyrine and Dexomethorphan.

15 Another aspect of the invention is a mixture comprising at least two probe compounds, all probe compounds being enriched with  $^{13}\text{C}$  and/or  $^{15}\text{N}$  NMR active nuclei for use as an agent for determining *in vivo* protein activity, whereby said determination is preferably used for phenotyping.

20 Yet another aspect of the invention is the use of a mixture comprising at least two probe compounds, all probe compounds being enriched with  $^{13}\text{C}$  and/or  $^{15}\text{N}$  NMR active nuclei for the manufacture of an agent for determining *in vivo* protein activity, whereby said agent is preferably used for phenotyping.

25 In a preferred embodiment, said mixture further comprises at least one putative drug. This mixture is preferably used evaluate potential drug-drug interaction.

30 In a preferred embodiment, said mixture comprises probe compounds which are substrates for CYP450, particularly preferably substrates for CYP450 isoenzymes selected from the group consisting of CYP1A2, CYP2A6, CYP2B6, CYP2C8/9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4.

In a further preferred embodiment, said mixture comprises probe compounds which are substrates for CYP450, particularly preferably substrates for CYP450 selected from the group consisting of Phenacetin, Coumarin, Tolbutamide, Mephenytoin, S-Mephenytoin, Bufuralol, Chlorzoxazone, Midazolam, Caffeine, Dapsone, 5 Diclofenac, Debrisoquine, Bupropion, Antipyrine and Dexomethorphan.

### Examples

#### 1) Selection of probe compounds

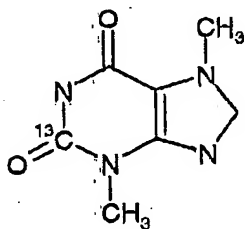
5

The activity of the CYP450 isoenzymes CYP1A2, CYP2D6 and CYP2E1 was determined using the following probe compounds :

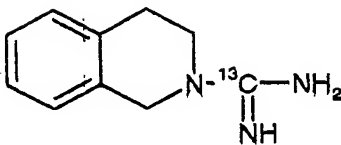
- Caffeine as a substrate for CYP1A2, caffeine is primarily metabolised to paraxanthine.
- 10 • Debrisoquine as a substrate for CYP2D6, debrisoquine is primarily metabolised to 4-hydroxy-debrisoquine.
- Chlorzoxazone as a substrate for CYP2E1, chlorzoxazone is primarily metabolised to 6-hydroxy-chlorzoxazone

15 The compounds used were isotopically labelled at the following positions:

Caffeine:

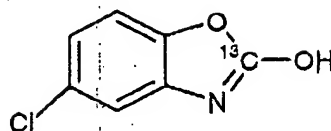


20 Debrisoquine:



25

## Chlorzoxazone



5

## 2) Administration of probe compounds and collection of samples

10 A SPD rat was sequentially injected interperitonally (i.p) with

- 43.3  $\mu\text{mol/kg}$  chlorzoxazone, solubilised in 250 $\mu\text{l}$  PEG 400,
- 36.9  $\mu\text{mol/kg}$  caffeine, solubilised in 10 mM sodiumphosphate buffer, pH 7.3, 0.9% NaCl and
- 41.5  $\mu\text{mol/kg}$  debrisoquine, solubilised in 10 mM sodiumphosphate buffer, pH

15

Urine samples were collected immediately before administration and then at 1 h and 2.5 h after administration. The volume of urine collected for each period was in the range of 1 to 10 ml. Blood samples were collected immediately before administration and then at 1, 2 and 3 h after administration. After collection, blood

20 samples were spun down at 2000 rpm for 10 min. and the plasma was collected. Both plasma and urine samples were frozen at  $-20^{\circ}\text{C}$ .

## 3) Hyperpolarisation of the collected samples and NMR analysis

25 A stock solution of tritylradical Tris(8-carboxyl-2,2,6,6-tetra(2-(1-hydroxyethyl))-benzo[1,2-d:4,5-d']bis(1,3)dithiole-4-yl)methyl sodium salt (428 mg, 300  $\mu\text{mol}$ ) in glycerol (12.61 g) was prepared. Aliquots of the stock solution (51.0 mg) were mixed with 40  $\mu\text{l}$  biofluid (urine or blood plasma) to give a 15 mM solution in trityl radical. These solutions were dispensed as droplets into liquid nitrogen to provide

30 the material as vitrified pellets suitable for hyperpolarisation. The solid samples were placed in turn within the DNP magnet and hyperpolarised overnight. The samples

were dissolved by injection of a mixture of methanol and acetic acid (100:1). The dissolved samples were quickly transferred manually (app. 4 s. transfer time) to a high-resolution magnet of 9.4 T and single acquisition  $^{13}\text{C}$ -1D-NMR-spectra were acquired.

5

#### 4) Results

A large number of signals were expected for the probe compounds and their multitudes of metabolites. In addition the biofluid matrix signals and the solvent signals were also expected to be present in the spectra.

For caffeine, major caffeine metabolites were expected to be 1,3-dimethyl uric acid; 1, 3, 7-trimethyl uric acid and paraxanthine. Further minor caffeine metabolites were expected to be 1-xanthine, 1, 3-xanthine; 3, 7-xanthine; 1, 3, 7-DAU; 3, 7-uric acid; 1, 7-uric acid and 1-uric acid. For debrisoquine, several metabolites including 4-hydroxy debrisoquine were present in addition to debrisoquine itself. In the urine sample collected after 3 h, only debrisoquine and 4-hydroxy debrisoquine were present. Additionally, two unknown signals were present which were possibly urine background signals.

#### 20 Quantification:

A known amount of the unlabelled probe compounds caffeine, chlorzoxazone, debrisoquine and their primary metabolites had been spiked into SPD rat urine samples, hyperpolarisation and NMR analysis was carried out as described above. From these experiments it was possible to estimate an enhancement factor for the carbon atoms of interest in the individual probe compounds and their metabolites. Quantification of the unknown amount of probe compound/metabolites in the biofluid samples was based on these enhancements and the known amount of probe compound/metabolite used to generate it. The carbon signal of interest was integrated and this integral value was adjusted (division by 90, 1.1% natural abundance  $^{13}\text{C}$ ) to account for the unlabelled probe compounds used in the spiked samples compared to the isotopically enriched probe compounds used in the biofluid samples. This integral was then related to the concentration of probe compound used in the spiked samples and compared to the integral obtained for the individual carbon signals identified in the biofluid samples. A careful phase and baseline correction

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had been performed before integration and the integral window was pre-adjusted to 25 Hz in all measurements. In case of signal overlap an estimated value had been obtained using the signal to noise ratio of the signals of interest.

The following concentration ranges were obtained:

5

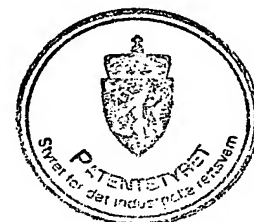
Urine	Sample collected after	Concentration range (µg/ml)
Caffeine	1 h	4 - 5
Caffeine	2,5 h	<3
Debrisoquine	1 h	<3
Debrisoquine	2,5 h	<2
4-OH-Debrisoquine	1 h	Below detection limit
4-OH-Debrisoquine	2,5 h	Below detection limit
Chlorzoxazone	1 h	>20, overlap; estimated based on SNR
Chlorzoxazone	2,5 h	>20, overlap; estimated based on SNR
6-OH-Chlorzoxazone	1 h	<3
6-OH-Chlorzoxazone	2,5	<3
<b>Plasma</b>		
Caffeine	1 h	5 - 7
Caffeine	2 h	5 - 10
Chlorzoxazone	1 h	Below detection limit
Chlorzoxazone	2 h	Below detection limit

In order to improve the detection limit, biofluid samples may be concentrated (e. g. by freeze-drying) before hyperpolarisation.

#### 10 Calculation of metabolic ratio:

The metabolic ratio is being used as a measure of the activity of an individual CYP450 isoenzyme and calculated as the percentage of unchanged probe compounds present in the biofluid sample divided by the percentage of metabolite. Thus, the calculation of the metabolic ratio of chlorzoxazone and its primary metabolite 6-

15 hydroxy-debrisoquine is used as a measure for the CYP2E1 activity, the metabolic ratio of caffeine and its primary metabolite paraxanthine is used as a measure for the CYP1A2 activity and the metabolic ratio of debrisoquine and its primary metabolite 4-hydroxy debrisoquine is used as a measure for the CYP2D6 activity.





### Claims

1. Method for determining *in vivo* protein activity comprising
  - 5 a) selecting at least two probe compounds each containing at least one NMR active nuclei
  - b) administering said probe compounds to a human or non human animate body
  - c) collecting samples from said human or non human animate body
  - d) hyperpolarising the NMR active nuclei of said samples, and
  - 10 e) analysing said samples by NMR spectroscopy
2. Method according to claim 1 wherein from said analysis of step e) an NMR pattern I is generated comprising the further steps of
  - 15 f) administering said probe compounds a) and in addition at least one putative drug to a human or non human animate body
  - g) subsequently carry out steps c) and d) according to claim 1
  - h) analysing the samples extracted in step d) by NMR spectroscopy and hereby generating an NMR pattern II
  - i) comparing the NMR patterns I and II and thereby identifying distinctions in the
  - 20 NMR pattern II which are due to the administration of the at least one putative drug.
3. Method according to claims 1 and 2 wherein the probe compounds are enriched with NMR active nuclei.
- 25 4. Method according to claims 1 to 3 wherein hyperpolarisation is carried out by means of polarisation transfer from a noble gas, brute force, dynamic nuclear polarisation (DNP) or spin refrigeration.
- 30 5. Method according to claims 1 to 4 wherein the probe compounds and/or the putative drugs are administered into the vasculature, an organ, tissue or via a non-parental route.
6. Method according to claims 1 to 5 wherein the collected samples are biofluids.

7. Method according to claim 1 to 6 wherein said probe compounds are substrates for Cytochrome P 450 (CYP450)
- 5 8. Method according to claim 7 wherein said probe compounds are substrates for CYP 450 isozymes selected from the group consisting of CYP1A2, CYP2A6, CYP2C8/9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4.
9. Method according to claims 1 to 8 for phenotyping
- 10 10. Method according to claims 2 to 8 for studying drug-drug interaction.
11. Mixture comprising at least two probe compounds, all probe compounds being enriched with  $^{13}\text{C}$ - and/or  $^{15}\text{N}$  NMR active nuclei.
- 15 12. Mixture according to claim 11 wherein said mixture comprises at least 3 probe compounds, preferably at least 4 probe compounds.
13. Mixture according to claim 11 and 12 wherein said probe compounds are
- 20 substrates for Cytochrome P 450 (CYP450)
14. Mixture according to claim 13 wherein said probe compounds are substrates for CYP 450 isozymes selected from the group consisting of CYP1A2, CYP2A6, CYP2B6, CYP2C8/9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4.
- 25 15. Mixture according to claim 13 and 14 wherein said probe compounds are selected from the group consisting of Phenacetin, Coumarin, Tolbutamide, Mephenytoin, S-Mephenytoin, Bufuralol, Chlorzoxazone, Midazolam, Caffeine, Dapsone, Diclofenac, Debrisoquine, Bupropion, Antipyrine and
- 30 Dexomethorphan.
16. Mixture according to claim 11 to 15 further comprising at least one putative drug.

17. Use of the mixture according to claims 11 to 16 for the determination of *in vivo* protein activity, preferably for pheotyping.
- 5 18. Use of the mixture according to claim 16 for studying drug-drug interaction.



5

Abstract

The invention relates to methods for determining protein activity using NMR spectroscopy. The present invention provides a method for determining protein activity *in vivo* using probe compounds and enhancing the nuclear polarisation of NMR active nuclei present in the probe compounds (hereinafter termed "hyperpolarisation") prior to NMR analysis. The invention also provides mixtures of probe compounds for the above-mentioned method.



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